Thio-phosphate can also be utilized by whole animals and/or complex tissues. Carassius auratus incorporates thio-phosphate into the DNA of intestinal cells; these cells are known to turnover in adults. Fish can be maintained in distilled water with NovAqua and AmQuel conditioners (US Patent 4,666,610). Goldfish flakes are also added for food. Normal phosphate levels in the aquarium are less than 1 mg/L (Hanna Phosphate Test Kit; Hanna Instruments). Fish are transferred to 100 ml of conditioned media with up to 250 mg/L of thio-phosphate added. No adverse reactions are seen in fish incubated for up to two days. Fresh media is prepared daily. Fish are sacrificed and DNA prepared from intestinal cells using DNAzol™ (Molecular Research Center, Ohio). The DNA samples are then tested for resistance to DNase digestion. Samples are incubated in a 50 ul rxn with ~ 1ug of DNA and .5 units of DNase for 0, 5, 10, 15 min. at room temp. DNA from 250 mg/L thio-phosphate media is much more resistant than wildtype DNA or DNA from 25 mg/L thio-phosphate media. Normal DNA and 25 mg/L thio-phosphate treated DNA is digested after 5 min. while 250 mg/L thio-phosphate treated DNA persists beyond 15 min. of digestion.

This information specifically addresses the examiners concerns regarding how to measure the level of phosphate in animal feed using a Hanna phosphate test kit. It also demonstrates using an excess of thio-phosphate over the normal phosphate levels in media enabling it to compete for incorporation. When the parent application was separated into 3 patents the issued parent underwent some modifications including adding a figure showing the goldfish data described in the original specification. This did not constitute new matter at prosecution and does not constitute new matter now.

Regarding Claim 10 USC 112 p. 1

Examiner indicates that Claim 10 steps 2 and 3 are not supported by the specification. Step 2 describes the importance of determining the optimal ratio of thio-phosphate to phosphate that maximally enhances protein synthesis. Maximal protein enhancement can be achieved at ratios less than 100% thio-phosphate and the optimal ratio varies with the organism. In the original specification a table Figure 4 was included showing how different ratios effected the level of protein synthesis. This Figure is now figure 2 (copy enclosed) of the present application. Figure legend is provided below from original specification.

FIG. 4 depicts a table summarizing the results of varying ratios of thio-phosphate to normal phosphate on gene specific expression in cultured organisms. A 100% ratio represents maximal substitution of the media with thio-phosphate. Arrows pointing upward represent enhanced expression relative to normal media whereas those pointing downward represent diminished expression. Thicker arrows correspond to a greater degree of change than thinner arrows. The approximate fold enhancement is indicated next the to arrow at peak expression.

An optimal ratio for AP enzyme expression in bacteria was found at 50%. In contrast optimal induction of protein synthesis in yeast occurred with as little as a 10% ratio of thio-phosphate to normal phosphate present in the media for at least two different proteins. The following is taken from the original specification.

The ability to modulate mRNA levels may greatly enhance the production of desired products. The degree of enhancement can be controlled by the ratio of thio-phosphate to phosphate used as well as through the addition of the modified phosphate **at various time points during the fermentation** process. In this regard, individual organisms vary in their requirement for phosphate. The optimal ratio of thio-phosphate to normal phosphate that enhances protein synthesis of a given gene product must be determined for a given organism. The optimal ratio may also vary under different cellular physiological states such as that imparted by the vector system employed (i.e. plasmid or phage) or growth phase (trophophase versus idiophase). While a generally optimal ratio for most genes may emerge for a given organism it is still possible that an unusually unstable mRNA may be preferentially induced at lower ratios of thio-phosphate to phosphate levels. If the organism can tolerate an increase in total cell protein than even stable mRNAs may be enhanced over time.

This section of the original specification (as well as the invention summary) describes how modulation of mRNA stability could be used to preferentially enhance the expression of particular mRNAs. The paragraph suggests that you can exploit the stability of unstable mRNAs relative to more stable mRNAs consistent with the mechanism by which thio-phosphate works. The less stable an mRNA is the greater the potential for its enhancement over other mRNAs. This is also seen in the data where a heterologous gene product (Bac AP gene expressed in yeast) is enhanced 10 fold in its protein expression. In this regard the Bac AP gene expressed in yeast was still enhanced at a 100% ratio where total protein synthesis is reduced. This demonstrates the preferential accumulation of this particular and presumably unstable mRNA species. The paragraph also suggests that for some organisms there may be a limit to the amount of protein expression you can achieve if protein synthesis factors become rate limiting and prevent a further increase in the amount of protein that can accumulate in the cell. The data supports this idea as at high ratios of thio-phosphate to phosphate protein synthesis declines. The data further supports this idea as well because bacterial cells form inclusion bodies at high protein concentrations so cells can have their limits. Total protein synthesis is presumably not directly proportional to total mRNA at high mRNA concentrations induced by the use of thio-phosphate.

The timing of induction or step 3 of claim 10 is also important. The above section of the specification indicates this in the bold highlighted text. The stability of mRNAs can vary with different physiological states and so you would logically want to stabilize the mRNA when it is being expressed and not when it is being degraded. This is supported by the section below taken again from the original specification. The idiophase occurs after the growth phase and the paragraph suggests to add thio-phosphate during the idiophase to enhance products made during then as opposed to the growth phase.

If the product is synthesized during the growth or the trophic phase then there are a number of possible points which may be rate limiting such as glucose uptake, glycolysis (often very fast), Kreb's cycle, enzymatic cataylsis or product feedback inhibition. Thus the rate of fermentation, duration, and/or yield of product may be increased depending on which step is rate limiting. If the product is produced during the idiophase as a secondary metabolite then mRNA stabilization of catalytic enzymes may also enhance yields during this later stage of fermentation. During the idiophase the modified phosphate can be simply added to the media to enhance mRNA stabilization.

The data from the present application demonstrate the addition to thio-phosphate during the growth phase as this is when the proteins tested are enhanced. If you added thio-phosphate after mRNAs were made you would see no effect. Also because thio-phosphate does decay as mentioned in the original specification it is best to add it only when it is needed.

The following paragraph from the original specification is also found to support step 2 of claim 10.

It is possible to increase the protein synthesis levels of many mRNAs through selective stabilization at optimal thio-phosphate to phosphate ratios. At very high ratios of thio-phosphate to phosphate total protein synthesis declines. At lower ratios where total protein synthesis is not affected, the levels of many typical and less abundant species is increased presumably at the expense of the most abundant species. The process of enhancement results from the preferential accumulation of proteins encoded by less stable mRNAs. The ratio of thio-phosphate to phosphate rather than the total phosphate level confers the degree of stabilization. In yeast it is known that many mRNAs for extracellular hydrolytic enzymes such as amylase are atypically stable with half-lives more than ten hrs. In contrast , most yeast mRNAs have half-lives on the order of 20 mins. Very low ratios of thio-phosphate to phosphate (10%) in yeast enhance the accumulation of the more typical mRNAs relative to the abundant and stable hydrolytic mRNA population. In bacteria, similar to yeast, beta-galactosidase is at least one abundant hydrolytic species that is reduced under conditions leading to an increase of other gene products. The degree of protein enhancement depends somewhat on the relative stability of corresponding mRNAs but can be as much as ten fold for less stable or heterologous gene products in bacteria and/or yeast.

I wish to declare and make a verified statement that the modified claims include no new matter as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825 (d).

Please under MPEP 707.07(j) the pro se applicant requests that if the Examiner fines patentable subject matter disclosed in this application, but feels that applicant's present claims are not entirely suitable, the Examiner draft one or more allowable claims for the applicant

Respectfully submitted,

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